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1 **The COP9 signalosome mediates transcriptional and metabolic response**
2 **for hormones, oxidative stress protection and cell wall rearrangement**
3 **during fungal development**

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1 **Summary**

2 The COP9 signalosome complex (CSN) is a crucial regulator of ubiquitin ligases. Defects in
3 CSN result in embryonic impairment and death in higher eukaryotes, whereas the filamentous
4 fungus *Aspergillus nidulans* survives without CSN, but is unable to complete sexual
5 development. We investigated overall impact of CSN activity on *A. nidulans* cells by
6 combined transcriptome, proteome and metabolome analysis. Absence of *csn5/csnE* affects
7 transcription of at least 15 % of genes during development, including numerous
8 oxidoreductases. *csnE* deletion leads to changes in the fungal proteome indicating impaired
9 redox regulation and hypersensitivity to oxidative stress. CSN promotes the formation of
10 asexual spores by regulating developmental hormones produced by PpoA and PpoC
11 dioxygenases. We identify more than 100 metabolites, including orsellinic acid derivatives,
12 accumulating preferentially in the *csnE* mutant. We also show that CSN is required to
13 activate glucanases and other cell wall recycling enzymes during development. These
14 findings suggest a dual role for CSN during development: it is required early for protection
15 against oxidative stress and hormone regulation and is later essential for control of the
16 secondary metabolism and cell wall rearrangement.

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1 Introduction

2 The conserved COP9 signalosome (CSN) multiprotein complex is present in higher
3 eukaryotic cells (Wei *et al.*, 1994). CSN controls light regulation, pathogen response,
4 pigment production and seedling survival in plants and is involved in regulation of tumor
5 growth, inflammation, hormone signalling, oxygen homeostasis and early development in
6 mammals. Defects in CSN result in embryonic lethality, presumably because CSN affects
7 various processes at the cellular level including the ubiquitin-proteasome pathway, DNA
8 damage response, cell cycle control and gene expression (Wei *et al.*, 2008). The main known
9 molecular function of CSN is the control of the activity of cullin-RING E3 ubiquitin ligases
10 (CRLs) conveyed by the metalloprotease-containing Csn5 subunit. CSN is able to regulate
11 CRLs by cleaving the ubiquitin-like peptide Nedd8 from lysine residues of cullins (Wu *et al.*,
12 2005). In addition, CSN-associated proteins mediate phosphorylation and deubiquitination of
13 substrates, including transcription factors such as plant HY5 (Osterlund *et al.*, 1999) or cJUN
14 (Naumann *et al.*, 1999) in animals affecting their stability and activity (Sun *et al.*, 2002,
15 Naumann *et al.*, 1999). CSN also controls transcriptional networks as a transcriptional co-
16 repressor of thyroid hormone receptor (Tenbaum *et al.*, 2003) and as a transcriptional
17 repressor during *Drosophila* development (Oron *et al.*, 2007). Fungi often possess incomplete
18 and non-essential versions of CSN, but the mold *Aspergillus nidulans* carries an eight-subunit
19 CSN holocomplex (Busch *et al.*, 2007). *A. nidulans* CSN is required for fruit body formation
20 but is not essential for filamentous growth or asexual sporulation (Busch *et al.*, 2003).
21 In contrast to simple modular vegetative hyphae, the development of sexual fruit bodies
22 results in a complex three-dimensional structure and requires formation of specialized cells
23 and tissues. *A. nidulans* fruit body formation can be induced in the dark by oxygen limitation
24 and is inhibited by light, which causes primarily airborne asexual spores to be formed
25 (Adams *et al.*, 1998, Purschwitz *et al.*, 2008, Bayram *et al.*, 2008a). CSN is essential for this

1 light-dependent control and its defects result in 'blind' organisms constitutively forming
2 initial sexual tissues. Mature *A. nidulans* fruit bodies are closed cleistothecia, developing
3 from primordia emerging from nests (Fig. 1A). Within these fruit bodies, meiotic cells are
4 enclosed by maternal envelope tissue and surrounded by nursing Hülle cells. CSN is required
5 for the light-dependent inhibition of fruit body formation, but also for its maturation, because
6 defective CSN results in a block in fruit body formation at the state of primordia (Busch *et*
7 *al.*, 2003).

8 In filamentous fungi, reactive oxygen species produced by NADPH oxidases provide a
9 specific trigger for sexual development (Lara-Ortiz *et al.*, 2003, Aguirre *et al.*, 2005).
10 Endogenous lipogenic signal molecules called psi (precocious sexual inducer) factors,
11 derived from linoleic acid, also play a role in regulation of spore development; the proportion
12 of different psi factors controls the ratio of asexual to sexual spore production (Tsitsigiannis
13 *et al.*, 2004a). The reorganization of vegetative hyphae to form the specialized cells and
14 tissues and ultimately the structure of the fruit body is accompanied by massive cell
15 proliferation. This process requires mobilization of energy and building materials, as well as
16 protection of the fruit body, coordinated by internal signaling. The energy accumulated
17 during growth in cell wall glucans has to be mobilized by degrading enzymes during
18 development, mostly α -1,3-glucanases such as MutA, expressed during sexual development
19 in Hülle cells (Wei *et al.*, 2001). Protecting the fruit body involves developmental
20 synchronisation of the synthesis of specific secondary metabolites including bioactive
21 molecules with the potential to repel fungivores (Bayram *et al.*, 2008b, Rohlfs *et al.*, 2007).
22 We took advantage of an organism possessing a complete non-essential CSN to investigate
23 the molecular mechanisms of CSN and its role in *A. nidulans* development by a first
24 combined transcriptome, proteome and metabolome analysis of this fungus. We found that
25 vegetative CSN functions necessary to handle oxidative stress are linked to development.

1 CSN is necessary in setting up the stage for the light-mediated decision between asexual and
2 sexual development by ensuring the appropriate ratio of psi factor hormone-like molecules.
3 CSN also appears to protect the fungus during the internal reactive oxygen species (ROS)
4 signalling triggering development. Furthermore, in absence of CSN we found dramatic
5 changes in secondary metabolism and reduced expression of glucanases necessary to
6 mobilize sugars stored in cell wall during development.

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1 **Results**

2 ***Fungal CSNE deneddylase is expressed during vegetative growth and early development***

3 *A. nidulans* CSN expression was estimated by monitoring the appearance of the CsnE/CSN5
4 deneddylase subunit during different developmental stages (Fig. 1B). A functional N-
5 terminal fusion of *csnE* to TAP tag (TAP-CsnE) was highly expressed in vegetative mycelia
6 and expression ceased by 48 h in developmentally induced tissues. Reduction of CsnE
7 protein levels coincided with the time point of the developmental block in Δcsn strains.
8 Developmental markers such as the catalase-peroxidase (CpeA-sGFP), expressed in Hülle
9 cells (Scherer *et al.*, 2002), only appeared at this time point (Fig. 1B), confirming that CSN
10 expression ceases after development is initiated. The pattern of CsnE expression implies that
11 CSN activity is restricted to vegetative mycelia and early development, prior to the
12 appearance of the Δcsn phenotypes.

13 CsnE is able to assemble the CSN holocomplex only when all other subunits are present
14 (Busch *et al.*, 2007), but the levels of CsnE do not depend on the presence of other subunits.
15 Expression of CsnE was monitored in strains lacking CsnA/CSN1 or CsnD/CSN4, which
16 showed the same developmental block as $\Delta csnE$ (Fig. 1C). We observed the same pattern of
17 TAP-CsnE expression independent on the presence of the complex. Whereas TAP-CsnE ran
18 as a single band in the presence of all other *csn* genes, additional lower molecular weight
19 bands appeared in strains lacking CsnA or CsnE. This suggests that an intact CSN complex
20 might prevent degradation of the CsnE subunit.

21

22 ***CSN affects transcription of 15 % of the fungal genome during development***

23 Microarrays of *csn* mutants in *D. melanogaster* revealed an achronic gene expression pattern
24 suggesting that CSN acts as a transcriptional co-repressor during development (Oron *et al.*,
25 2007). We compared fungal transcriptomes of wild type and $\Delta csnE$ mutant strains during

1 development to monitor the effects of CSN on gene expression at different developmental
2 stages. Four time points were compared pairwise between both strains (Fig. S1): after 14 and
3 20 hours of vegetative growth in liquid culture (V14 and V20 respectively, before and after
4 achieving developmental competence) and after 48 h of growth on solid medium, induced to
5 develop either asexually in light (A48) or sexually in darkness (S48). At these timepoints
6 mutant and wild type morphologies were similar (Fig. 2A, B).

7 The microarray data derived from two biological and four technical replicates revealed that
8 the expression levels of 15.4 % of 10,546 annotated *A. nidulans* genes were strongly (\log_2
9 ratio ≥ 3.0 and adjusted p value ≤ 0.01) or moderately (\log_2 ratio ≥ 2 and adjusted p value \leq
10 0.01) altered in the mutant throughout development (Table S1). The expression of 1,256
11 genes, representing 11.9 % of the genome, was moderately altered, and expression of 369
12 genes, representing 3.5 % of the genes on the array, was strongly altered at least at one
13 developmental stage. The transcript levels of the remaining seven CSN subunits were similar
14 in wild type and $\Delta csnE$ (data not shown). Only the genes showing strongly altered expression
15 were analyzed further.

16 Differentially expressed genes were assigned to 11 functional categories based on available
17 annotation or similarities to characterized proteins from other organisms (Fig. 2C). During
18 vegetative growth, the most changes in the $\Delta csnE$ mutant were observed among genes
19 involved in secondary metabolism and redox reactions. In developing cultures, CSN
20 repressed many genes playing a role in protein degradation, stress response and defense, cell
21 wall biosynthesis as well as redox reactions, which were mainly upregulated in the mutant.
22 At the same time, CSN activated numerous genes involved in secondary metabolism and cell
23 wall degradation, accordingly downregulated in the mutant.

24 During vegetative growth CSN acts mainly as a transcriptional activator, with two to three
25 times (at V20 and V14, respectively) as many genes downregulated than upregulated in the

1 $\Delta csnE$ mutant. Under development-inducing conditions, the number of up- and
2 downregulated genes was similar (Table S1). More than half of the genes differentially
3 expressed during vegetative growth showed parallel regulation at both time points; notably,
4 twice as many genes were downregulated in $\Delta csnE$ at V14 than at V20. Thus, the
5 transcriptome underwent significant changes in the absence of the COP9 signalosome even
6 before achieving developmental competence by the fungus. This data does not support an
7 exclusive transcriptional corepressor function of CSN, but suggests its involvement in control
8 of both transcriptional activation and repression. Although CSN is expressed only during the
9 early stages of development (until 24 h), the largest changes in the transcriptome were
10 observed later in development (at 48 h), indicating delayed downstream effects on gene
11 expression.

12

13 ***csnE deletion leads to changes in protein production reflecting impaired redox regulation***

14 CSN-mediated deneddylation regulates the activity of cullin-based ubiquitin ligases, which
15 should be reflected by changes in the stability of proteins directed for degradation in the
16 proteasome. To examine this posttranscriptional role of CSN, we examined the changes in the
17 proteome of the $\Delta csnE$ strain during vegetative growth in the time window when
18 developmental competence is acquired (Fig. 3). After 2D gel electrophoresis of soluble
19 protein extracts, ten spots representing eight unique proteins with significant differences in
20 their intensities between the two strains were identified by mass spectrometry (Table S3).

21 Deletion of *csnE* significantly increased the levels of five proteins (Fig 3). They include four
22 metabolic enzymes involved in carbon and amino acid metabolism. The branched-chain
23 amino acid biosynthetic enzyme ketol-acid reductoisomerase and carbon metabolism enzyme
24 pyruvate decarboxylase were described as oxidative stress targets in fungi (Cabiscol *et al.*,
25 2000, Reverter-Branchat *et al.*, 2004, Yoo & Regnier, 2004). S-adenosylmethionine (SAM)

1 synthetase is required for biosynthesis of cysteine, which is a precursor of the antioxidant
2 glutathione. HMG-CoA synthase is a key enzyme in the mevalonate pathway required for
3 synthesis of many primary and secondary metabolites. The putative monooxygenase is
4 thought to be involved in detoxification reactions or complex metabolite modifications
5 (Harayama *et al.*, 1992).

6 The levels of three proteins are decreased in the *csnE* deletion mutant. The first two, actin
7 and elongation factor 2 (EF-2) have also been described as targets of oxidative stress
8 (Cabiscol *et al.*, 2000, Reverter-Branchat *et al.*, 2004, Yoo & Regnier, 2004). The third
9 protein is a peroxiredoxin, mediating protection against oxidative stress; peroxiredoxins are
10 important cellular antioxidants identified in a wide range of organisms (Rhee *et al.*, 2005).

11 This proteome comparison reveals an effect of CSN on the levels of cytoplasmatic proteins
12 sharing a connection to oxidative stress. Although putative stress-related genes were also
13 affected by CSN at the transcriptional level, there was almost no correlation between
14 differentially expressed genes and proteins during vegetative growth. This is not surprising as
15 the changes in the proteome are likely due to a direct impact of CSN on protein stability,
16 whereas the transcriptional regulation probably reflects a role of CSN in regulating the
17 corresponding transcription factors. A notable exception is the putative monooxygenase,
18 which is less abundant at both transcript and protein level in the $\Delta csnE$ strain and might be
19 worth investigating further.

20

21 ***CSN controls stress and defense genes making the $\Delta csnE$ strain sensitive to redox***
22 ***imbalance***

23 Comparative analysis of the vegetative transcriptome and proteome suggested a significant
24 impact of CSN on stress and defense-related processes. The largest group of genes affected in
25 the $\Delta csnE$ strain encode putative oxidoreductases of primary or secondary metabolism (Fig.

1 2C, Table S4). Genes encoding proteins of known enzymatic function were mostly assigned
2 to the primary or secondary metabolism functional categories. Therefore the ‘redox enzymes’
3 category comprises mostly oxidoreductases where the molecular function remains to be
4 determined. The transcriptome data suggests that CSN affects some of these genes during
5 vegetative growth and others primarily during development.

6 Northern hybridization of four oxidoreductase transcripts verified this phase-specific
7 transcriptional regulation (Fig. 4). CSN induces developmental transcription of *catD* gene,
8 encoding catalase D required for oxidative stress response and expressed in Hülle cells
9 (Aguirre *et al.*, 2005). It also activates transcription of a putative defensin gene primarily
10 during vegetative growth. On the other hand, CSN represses a tyrosinase gene involved in
11 protective melanin production during vegetative growth and a gene encoding a putative
12 secondary metabolism oxygenase throughout the life cycle (Fig. 4, Table S4).

13 These changes in gene expression translate to marked differences in response to oxidative
14 stress between wild type and $\Delta csnE$ strains (Fig. 5A). $\Delta csnE$ is more sensitive to menadione
15 (which increases cellular levels of ROS) and to the thiol-oxidizing drug diamide causing
16 redox imbalance. Diamide shifts the cellular ratio of reduced glutathione (GSH) to oxidized
17 glutathione (GSSG). Accordingly, GSSG had a greater limiting impact on growth of the
18 $\Delta csnE$ strain than GSH. Treatment with either GSH or GSSG resulted in concentration-
19 dependent appearance of red colouring in the growth medium of the mutant, and to a lesser
20 extent in the wild type. It had been described earlier that aging *csn* mutant colonies also
21 accumulate a red pigment even in the absence of any redox stress (Busch *et al.*, 2003). Under
22 conditions inducing asexual development, low GSH concentrations led to increased number
23 of sexual structures in all strains, whereas higher concentrations increased asexual
24 conidiation (data not shown). The growth of the $\Delta csnE$ strain was also impaired on medium

1 supplemented with 4% ethanol as an inducer of oxidative stress (data not shown).
2 Furthermore, $\Delta csnE$ was sensitive to increased temperature (Fig. S2).
3 A functional CSN deneddylase is required for efficient redox and temperature stress response
4 of *A. nidulans*. Both transcriptome and proteome analysis support a strong connection
5 between oxidative stress and fungal development and point towards CSN-mediated
6 regulation of catalase D as well as many still uncharacterized oxidoreductases in this process,
7 ensuring resistance to a wide variety of oxidising and other stress factors.

8
9 ***Loss of CSN leads to significant changes of the fungal metabolome, inducing***
10 ***accumulation of orsellinic acid-derived metabolites and sterigmatocystin intermediates***

11 We investigated the chemical composition of compounds producing the red colour released
12 to the growth medium in the mutant to explore the link between oxidative stress and the
13 mutant phenotype. The yield of the compounds increased by cultivating the wild type and
14 $\Delta csnE$ strains on the surface of liquid medium for ten days. Whereas several high polymeric
15 compounds could not be identified, extraction of culture filtrates with ethylacetate and thin-
16 layer chromatography revealed many more unique red-coloured substances in the $\Delta csnE$
17 mutant than in the wild type (Fig. 5B). Strains grown on ammonium instead of nitrate as a
18 nitrogen source produced highest yields. Pure compounds were isolated and five orsellinic
19 acid-related metabolites could be identified: orcinol, diorcinol, cordyol C, violaceol I, and
20 violaceol II (Fig. 5C, S3, S4). Four colourless substances were obtained, probably undergoing
21 polymerization to produce yellow, orange or red coloured substances when dissolved in
22 methanol.

23 The archetypal polyketide orsellinic acid is produced by enzymes encoded by a recently
24 discovered *A. nidulans* gene cluster (Schroeckh *et al.*, 2009). Indeed, several orsellinic acid
25 synthesis genes are differentially expressed in the $\Delta csnE$ mutant (Table S4). *orsB*, encoding

1 an amidohydrolase and *orsC*, encoding a tyrosinase are upregulated in the $\Delta csnE$ strain under
2 asexual conditions and *orsB* also under sexual conditions. In contrast, the *orsA* alcohol
3 dehydrogenase gene is repressed during late vegetative growth in the mutant (V20). This
4 suggests that the production of these secondary metabolites by the mutant is mainly a result
5 of transcriptional derepression.

6 A more comprehensive untargeted metabolite fingerprinting analysis was then performed at
7 different time points to investigate intracellular compounds. Overall, the analysis revealed
8 more than 100 metabolite markers differing in their occurrence at least at one developmental
9 stage between wild type and $\Delta csnE$ mutant. Intensity-based clustering by one-dimensional
10 self-organizing maps (1D-SOMs) (Meinicke *et al.*, 2008) was used to get an global overview,
11 shown here for marker candidates of the non-polar extracts (negative ionization mode). The
12 intensity profiles of the marker candidates are represented by 20 prototypes separated in
13 blocks of different intensity patterns (Fig. 6A). A first dominant block represents $\Delta csnE$ -
14 specific marker candidates, which accumulate mainly under asexual (prototypes 6-8) or
15 sexual conditions (prototype 13) in the mutant. A second block (prototypes 16-20) contains
16 developmental markers specific for the wild type and downregulated in $\Delta csnE$ strain.
17 Prototypes 10-12 and 14-15 can be combined to a block representing mutation-independent
18 markers specific for asexual and/or sexual development.

19 Prototype 7 revealed several markers, identified as intermediates of the sterigmatocystin (ST)
20 biosynthetic pathway and strongly accumulated in the $\Delta csnE$ strain at A48 time point (Fig.
21 6B, Table S6). Accordingly, transcription of genes of ST and ST-like pathways, presumably
22 involved in the biosynthesis of polyketide secondary metabolites (Brown *et al.*, 1996), was
23 upregulated in the mutant at this time (Table S4). Fig 6C summarises the correlation between
24 $\Delta csnE$ metabolome data and the corresponding upregulated genes of the *A. nidulans* ST gene
25 cluster: *stcE* encodes a putative norsolorinic acid reductase producing averantin (Brown *et*

1 *al.*, 1996), *stcW* encodes a monooxygenase synthesizing 1'-hydroxyversicolorone (Keller *et*
2 *al.*, 2000), the *stcU*-encoded versicolorin reductase catalyses demethyl-ST production, and
3 *stcV* encodes another norsolorinic acid reductase with a predicted role in reduction of
4 averufanin. The function of *stcC* is still unknown.

5 We next analysed secretion of sterigmatocystin to the growth medium and found that the
6 $\Delta csnE$ mutant produced significantly less ST than wild type (Fig. S5). It seems that
7 transcriptional *stc* gene upregulation in the mutant (Table S4) resulted in an accumulation of
8 ST intermediates (Fig. 6B), but also in a defect in production and secretion of the end
9 product, which normally protects developmental structures against threatening organisms in
10 the soil.

11

12 ***CSN controls the ratio of psi factors acting as A. nidulans sexual hormones***

13 Light normally stimulates formation of asexual spores and blocks sexual development in wild
14 type *A. nidulans*, but not in $\Delta csnE$. Two genes, *ppoA* and *ppoC*, encode antagonistic
15 dioxygenases involved in biosynthesis of psi factors, lipogenic developmental hormones. A
16 subtle balance between different psi factors is required to shift between developmental
17 programs (Tsitsigiannis *et al.*, 2004b). *ppoA* is necessary for formation of sexual and *ppoC* of
18 asexual spores. Both overexpression of *ppoA* and deletion of *ppoC* shifts the developmental
19 balance towards sexual sporulation (Tsitsigiannis *et al.*, 2004a). *ppoC* expression is necessary
20 already during vegetative growth to enable normal development, but it is present at much
21 higher levels present in light (at A48) than in dark (at S48), which is necessary to induce
22 asexual development in the wild type (Fig. S6). The $\Delta csnE$ mutant showed a strong
23 upregulation of the *ppoA* gene in light (at A48), accompanied by a reduction in *ppoC*
24 expression (Table S4) not only during development but also in early vegetative growth phase,
25 prior to any visible morphological change (Fig. 4, Table S4).

1 We went on to investigate whether these differences in *ppoA* and *ppoC* expression are
2 affecting the actual psi hormone factor levels, which would confirm an early role of CSN in
3 regulation of psi factor biosynthesis (Figs. S6 and S7). Three oxylipins, the PpoA products 8-
4 hydroxy-9-octadecanoic acid (8-HOE) and 8-hydroxy-9,12-octadecadienoic acid (8-HOD)
5 (Brodhun *et al.*, 2009a) as well as the PpoC product 10-hydroxy-9,12-octadecadienoic acid
6 (10-HOD) (Brodhun *et al.*, 2009b) could be detected in cultures. Concentration of PpoC-
7 dependent 10-HOD required for asexual sporulation is reduced at all time points in the
8 absence of CsnE, which is the most likely cause of the increased initiation of sexual
9 structures by the mutant (Fig. 7A). Concentration of PpoA-derived 8-HOD is similar in wild
10 type and mutant strains during vegetative growth, but reduced in the mutant during
11 development (Fig. 7B), whereas levels of 8-HOE are reduced in the mutant during late
12 vegetative growth and asexual development (Fig. 7C). The ratio of both PpoA-derived psi
13 factors, 8-HOE and 8-HOD, is important for the balance between sexual and asexual spore
14 development (Tsitsigiannis *et al.*, 2004b). Sexual and asexual development are characterized
15 by different 8-HOE to 8-HOD ratios; 1: 8.3 under sexual conditions as compared to 1: 2.7
16 under asexual conditions (Table 1). High *ppoA* expression in the $\Delta csnE$ mutant perturbs this
17 balance resulting in a constantly high 8-HOD proportion both in light (1:18.7) and in
18 darkness (1:15.1), which is likely the driving factor behind uncontrolled initiation of sexual
19 structures.

20 Our data indicates that CSN is required prior to initiation of development during vegetative
21 growth, as well as at early asexual developmental stages, to ensure the right balance of sexual
22 hormones. This is critical for the expression of the master transcriptional regulator of asexual
23 sporulation *brlA*, which was indeed downregulated in the mutant in light (Table S4). The
24 *brlA*-dependent *wA* encoding a polyketide synthase producing the conidial yellow pigment
25 was also downregulated (Table S4).

1 *CSN controls the activation of cell wall degrading enzymes including β -glucanases*

2 The main metabolic transcriptional networks involved in carbon metabolism were not
3 significantly affected in the $\Delta csnE$ mutant. However, expression of a large group of genes
4 associated with cell wall degradation was very strongly dependent on a functional CSN. They
5 were mainly downregulated in the mutant after the initiation of development, comprising
6 21% and 39% of all downregulated genes at A48 and S48, respectively (Table S2, Table S5).
7 This group of genes encode chitinases, glucanases and enzymes involved in degradation of
8 plant cell wall material, such as cellobiose hydrolases (Table S5). Strikingly, 32 of the 37
9 glycosyl hydrolases present in the *A. nidulans* genome were differentially expressed at least
10 at one developmental stage and 14 were downregulated during development. Northern
11 hybridization confirmed that $\Delta csnE$ was unable to express the cell wall degrading α -
12 glucanase gene *mutA* during sexual development (Fig. 4B). Surprisingly, only one more α -
13 glucanase gene was affected, while CSN had most impact on genes encoding putative β -
14 glucanases with a potential to hydrolyse both β -1,3- and β -1,4-glucans (Table S5).
15 Laminarinase (β -1,3-glucanase) activity was reported to increase in developing cultures
16 (Zonneveld, 1975, Bagga *et al.*, 1989), but there was no indication up to date that it is
17 required for sexual development.

18 We measured cytoplasmic laminarinase activity and confirmed that it was first induced after
19 48 h of development. The presence of CsnE was necessary for this induction during both
20 asexual and sexual development (Fig. S8). During metabolite fingerprinting we detected
21 accumulation of various disaccharides, including maltose, galactose, galactosamine and N-
22 acetylgalactosamine during sexual and asexual development in the wild type strain, most
23 likely as a result of high glucanase and chitinase gene expression (Table S6). Developmental
24 downregulation of α - and β -glucanases in the $\Delta csnE$ mutant, concomitant with upregulation
25 of a chitin synthase gene (Table S5), resulted in a reduced accumulation of disaccharides.

1 These findings reveal the importance of CsnE in regulating accumulation of cell wall-
2 derived sugars acting both as a reserve for energy-consuming developmental processes and
3 materials for cell wall remodeling.

4 CSN-dependent cellular remodelling also involves membrane transport, which is apparent by
5 major changes in expression of membrane transport related genes induced by *csnE* deletion
6 (Fig. 2C). The second largest group of genes differentially expressed at all developmental
7 stages (up to 8 %) fell into this 'transport' category. They encoded proteins facilitating
8 membrane transport of different metabolites, predominantly carbohydrates, amino acids and
9 toxins. This might be an additional reason why only the wild type accumulated β -glucan and
10 chitin decomposition products, which provide building blocks for restructuring cell wall
11 during development.

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1 Discussion

2 The underlying cause of embryonic lethality in higher eukaryotes deficient in CSN is a
3 crucial question in the field of COP9 signalosome research. We used a filamentous fungus
4 which can survive without CSN, but is impaired in sexual development as a model to
5 investigate the role of COP9 signalosome in development. This work represents the first
6 comprehensive genome-wide transcriptome, proteome and metabolome analysis of both the
7 COP9 signalosome function and of fungal development. As a result we were able to identify
8 multiple processes depending on an intact CSN during the differentiation of the complex
9 multicellular structure of the fruit body (Fig. 8). It is feasible that the role in some of these
10 processes, which are conserved on a molecular level in multicellular eukaryotes, is making
11 CSN indispensable for plant and animal development. The presence of CSN is required to
12 ensure resistance to oxidative stress, the right concentration of sexual hormones, protection of
13 the fruit body (including the production of toxic secondary metabolites) and mobilization of
14 cell wall glucans during development.

15 A connection between CSN and oxidative stress response was observed in higher eukaryotes,
16 where CSN5 binding partners accumulate under hypoxic conditions. CSN5 associates with
17 several redox-sensitive transcription factors such as AP-1 (Abate *et al.*, 1990), NF- κ B (Galter
18 *et al.*, 1994), p53 (Bech-Otschir *et al.*, 2001) and with the antioxidative enzyme thioredoxin
19 (Hwang *et al.*, 2004). Plant seedlings of CSN mutants accumulate the antioxidant
20 anthocyanin which is upregulated by high levels of light-induced ROS (Dohmann *et al.*,
21 2005). Protection against oxidative stress is required for fungal development, because a
22 transient increase in ROS levels acts as an internal signal for developmental progression
23 (Aguirre *et al.*, 2005). This resistance, involving the expression of *catD* catalase gene (Fig.
24 4), is dependent on the COP9 signalosome, and while not essential for normal growth it
25 seems to set the stage for development by allowing the cell to quench the ROS signal and

1 avoid oxidative damage. ROS-mediated signaling balanced by protection conferred by
2 endogenous antioxidants is emerging as a shared feature in the regulation of multicellular
3 development (Covarrubias *et al.*, 2008, Matsuzawa & Ichijo, 2008).

4 The *A. nidulans csnE* mutant strain develops Hülle cells, but cannot develop sexual structures
5 beyond the stage of primordia. Hülle cells have a nursing function and supply the developing
6 fruit bodies with nutrients. Developmental progression is also accompanied by specific
7 expression of catalase peroxidase and NADPH oxidase (NoxA) in Hülle cells (Scherer *et al.*,
8 2002). These enzymes lead to the production of superoxide and H₂O₂. Genes for Nox
9 enzymes are restricted to fungi with the potential for fruit body formation, but are common in
10 higher eukaryotes. Fungal $\Delta noxA$ strains are blocked at the same primordial state (Lara-Ortiz
11 *et al.*, 2003) as *csn* deletion strains. Thus, the most apparent effect of CSN is involved in the
12 only ROS-dependent developmental transition known so far in fungi. In mammalian
13 development, the first ROS-induced programmed cell death occurs in the blastocyst, causing
14 apoptosis of inner cells destined to develop into trophoctoderm (Parchment, 1993).
15 Strikingly, mouse embryos lacking *Csn5* survive to the blastocyst state but die soon after
16 implantation without undergoing gastrulation (Tomoda *et al.*, 2004) suggesting a
17 corresponding involvement of COP9 in ROS-mediated signaling. During the acquisition of
18 developmental competence in *A. nidulans* a protective peroxiredoxin PrxB (AN10223) is
19 expressed in emerging Hülle cells of wild type, but not of the $\Delta csnE$ strain (data not shown).
20 The PrxB murine homologue Prdx6 plays an important role in antioxidant defense in
21 the lungs and protects the lens epithelium from UV-induced cell death (Manevich & Fisher,
22 2005, Kubo *et al.*, 2010). It would be interesting to see if mammalian Prdx6 is also targeted
23 by CSN.

24 Impaired oxidative stress response resulting in the accumulation of unusual secondary
25 metabolites becomes apparent in the $\Delta csnE$ mutant already during vegetative growth.

1 Oxidative stress responses require catalase, superoxide dismutase and peroxiredoxin enzyme
2 activities as well as thioredoxin and glutathione as redox buffering systems (Aguirre *et al.*,
3 2005). Increased levels of S-adenosylmethionine synthetase (required for glutathione
4 biosynthesis) and of HMG-CoA synthase levels in the mutant suggest increased isoprenoid
5 biosynthesis presumably to produce protective carotenoids (Iigusa *et al.*, 2005), providing an
6 additional indication of elevated oxidative stress (Fig. 3).

7 The red-coloured compounds conspicuously released into the medium by the *csn* mutant are
8 more complex than we had anticipated (Fig. 5, S3, S4). They include several orsellinol- and
9 orsellinic acid-derived compounds that have only recently been assigned to *A. nidulans* when
10 cocultivated with a competitor organism, *Streptomyces hygroscopicus* (Schroeckh *et al.*,
11 2009). Production of these metabolites depends on a physical interaction of *A. nidulans* and
12 *S. hygroscopicus* and comprises a putative defense mechanism induced by the exchange of
13 reactive oxygen species. Secretion of these phenolic compounds by the hypersensitive mutant
14 could also be interpreted as a visible constitutive reaction to oxidative stress. Moreover,
15 gene expression of several oxidoreductases and a polyketide synthase in several yet
16 unexplored gene clusters involved in the production of unknown secondary metabolites is
17 upregulated in the $\Delta csnE$ strain (data not shown), suggesting an even broader fungal
18 metabolic repertoire in response to stress.

19 The presence of CSN during vegetative growth is prerequisite for developmental processes
20 accompanied by rearrangement of the cell wall. This requires the activity of numerous cell
21 wall degrading enzymes and a major adjustment of the membrane transport systems to shuttle
22 metabolites. $\Delta csnE$ strain showed a marked decrease in expression of genes involved in cell
23 wall and membrane-associated functions (Table S5), indicating that a functional CSN is
24 required for cell wall remodelling. In particular, it is necessary for induction of β -glucanase
25 activity targeting β -1,3-glucans and possibly also β -1,4-glucans (Fig. S8), which appear to be

1 mobilized along with α -glucans during development. The accumulation and subsequent
2 degradation of cell wall α -glucans is necessary for fruit body formation in *A. nidulans*
3 (Zonneveld, 1972, Zonneveld, 1974). β -glucans belong to an alkali-insoluble fraction of the
4 *Aspergillus* cell wall and were thought to be responsible mostly for its structural framework.
5 Production of endo- β -1,4-glucanases is induced during cleistothecial development (Bagga *et*
6 *al.*, 1989), but a requirement for β -glucanase activity for fruit body formation had not been
7 described. The extent of cell wall remodelling necessary for fruit body formation might
8 therefore be much greater than previously anticipated.

9 Other genes involved in cell wall remodelling are also affected in the Δ *csnE* mutant, which is
10 consequently unable to accumulate decomposition products of α - and β -glucans as well as of
11 chitins during development. Concomitant upregulation of putative genes responsible for
12 synthesis of α -glucan and chitin suggests that CSN is either directly or indirectly required for
13 cell wall recycling during development at many levels. Accumulation of mono- and
14 disaccharides which is impaired in the mutant (Table S6) might be an early trigger for the
15 initiation of later steps of sexual development that are blocked in the Δ *csnE* mutant.

16 Synthesis of secondary metabolites by fungi is intimately related to development; toxins such
17 as ST have been proposed to protect against fungivores (Rohlf *et al.*, 2007). CSN appears to
18 be required for production of secondary metabolites coinciding with development. The Δ *csnE*
19 mutant produces almost no ST, which is accompanied by a misregulation of genes in the ST
20 biosynthesis pathway and an accumulation of various ST intermediates during development.
21 CSN was also necessary for the expression of genes encoding cell wall proteases (Table S5)
22 and protective structural components of the spore wall, mainly hydrophobins (Table S7).
23 Taken together, these data suggest that CsnE is crucial in coordination of multiple pathways

1 which ensure the protection of newly produced sexual structures against various external
2 threats.

3 *A. nidulans* $\Delta csnE$ strain is unable to complete and regulate sexual development, and
4 constitutively develops sexual Hülle cells and primordia. CSN-dependent secondary
5 metabolites also include psi factors, hormone-like oxylipins, which have direct sporogenic
6 effects and regulate the balance of asexual and sexual spore formation in *A. nidulans*. Control
7 of psi synthesis depends on at least two *ppo* genes encoding psi factor-producing fatty acid
8 oxygenases (Tsitsigiannis *et al.*, 2005). PpoA and PpoC have antagonistic functions, whereby
9 PpoC ultimately promotes asexual and PpoA sexual sporulation (Brodhun *et al.*, 2009a). CSN
10 is required for the expression of *ppoC* in early vegetative stage, which probably contributes to
11 the mutant's inability to inhibit sexual development under asexually inducing conditions such
12 as light. Indeed, misregulation of *ppoA* and *ppoC* results in an imbalance of psi factor
13 biosynthesis. Constitutive *ppoC* downregulation is reflected by permanently low levels of the
14 PpoC-derived 10-HOD during vegetative growth (Fig. S7). Decreased 10-HOD concentration
15 at an early vegetative stage, when developmental competence is not yet achieved, seems to be
16 the underlying cause of the light insensitive phenotype of the mutant. Moreover, the ratio of
17 the PpoA-derived psi factors 8-HOE to 8-HOD is essential for the timing and balance of
18 asexual and sexual differentiation (Tsitsigiannis *et al.*, 2004a). The lower the ratio, the more
19 the developmental balance shifts towards asexual differentiation. Constitutively increased
20 *ppoA* expression in the mutant results in the distortion of the 8-HOE:8-HOD ratio (Table 1),
21 further enhancing the shift of developmental balance towards sexual differentiation.

22 The effect of CSN on hormone signals could account for the lack of transcriptional activation
23 of the asexual master regulatory gene *brlA* by light in the $\Delta csnE$ mutant. The BrlA
24 transcription factor initiates a cascade of additional regulatory genes including the AbaA
25 transcription factor which further activates *brlA* and numerous downstream genes

1 (Andrianopoulos & Timberlake, 1994). CSN is known as a negative regulator of stability of
2 transcription factors in other systems, such as JY5 in plants and p53 in mammals. The
3 stability of these proteins is modulated by CSN-regulated degradation by the ubiquitin system
4 (Bech-Otschir *et al.*, 2001, Schwechheimer *et al.*, 2002). It is currently unknown whether
5 CSN regulates the stability or activity of developmental transcription factors in *A. nidulans*.
6 However, genes encoding components of the ubiquitin system were upregulated in the mutant
7 during development and include several putative F-box proteins (Table S7, Fig. 4A), which
8 convey target specificity in SCF ubiquitin ligases, a prototypical group of cullin-RING E3
9 ubiquitin ligases (CRLs). Analysis of promoter regions of genes downregulated during
10 development in the $\Delta csnE$ mutant revealed statistically overrepresented binding sites for
11 AbaA transcription factor (Andrianopoulos & Timberlake, 1994); McmA, a homologue of
12 yeast transcription factor Mcm1 involved in sexual development (Passmore *et al.*, 1989); and
13 stress response elements (STREs) interacting proteins (Marchler *et al.*, 1993) (Fig. S6). These
14 factors do not appear to be regulated by CSN on a transcript level, suggesting that it might be
15 acting instead by regulating their protein stability, which remains to be investigated
16 experimentally.

17 We assume that most of the effects observed in the fungal $\Delta csnE$ mutant result from the lack
18 of deneddylase activity, removing Nedd8 ubiquitin-like peptide from CRLs. Deneddylation
19 regulates the activity of CRLs, which are disassembled and can be reassembled only after
20 another round of neddylation (Cope *et al.*, 2002). We hypothesise that the wide scope of
21 vegetative and developmental CSN functions in *A. nidulans* reflects the regulation of various
22 CRLs fulfilling regulatory roles in different regulatory networks and time points. As
23 additional function, it has been shown in *N. crassa* that CSN is also involved in the circadian
24 clock (He *et al.*, 2005). The identity of neddylated proteins involved in the control of *A.*
25 *nidulans* development and secondary metabolism remains to be explored. Another open

1 question is whether CSN-associated proteins mediating phosphorylation and deubiquitination
2 of substrates are also involved in these processes (Sun *et al.*, 2002, Naumann *et al.*, 1999).
3 In summary, we demonstrate that CSN exerts a broad role, both during vegetative growth
4 when it is crucial for determining later developmental fate and during development itself. Our
5 data suggest a much greater extent of cell wall remodelling prior to fruit body formation than
6 previously recognized in fungi. The broad metabolic repertoire of CSN-deficient strain
7 confirms the fungal potential for producing novel secondary metabolites under stress revealed
8 by recent research. Our data also provide evidence for a crucial biological role of CSN in
9 regulating redox homeostasis of eukaryotic cells, especially during processes dependent on
10 ROS signaling. Combined deficiencies in these functions, although not essential for
11 vegetative growth of a single cell or modular organism under laboratory conditions, might be
12 the reason why the development of multicellular plant or mammalian embryo, or a fungal
13 fruit body cannot be completed. Our comprehensive analysis opens further avenues into
14 research on the molecular functions of the COP9 signalosome in filamentous fungi, both as a
15 way to explore novel natural products and as a model of mammalian development.

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2 **Materials and methods**

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4 ***A. nidulans* culture**

5 *A. nidulans* strains were cultivated at 37 °C or 30 °C in minimal medium and fungal
6 development was induced as described (Clutterbuck, 1974). Briefly, asexual sporulation was
7 induced by incubation in continuous white light and cleistothecia formation by oxygen-
8 limiting conditions on plates sealed with cellophane film grown in the dark. Oxidative stress
9 assays were performed with 4% ethanol, menadione (0.04, 0.06, 0.08, 0.10 mM), diamide
10 (0.8, 1.2, 1.4, 1.6 mM), GSH or GSSG (5, 10, 15, 20 mM), added to the medium. Growth,
11 conidiation and cleistothecia development of *A. nidulans* was observed by differential
12 interference contrast microscopy (DIC).

13

14 ***Microarray RNA extraction, labelling and hybridisation***

15 RNA was extracted from 2 ml of ground fungal mycelium with Trizol (Invitrogen), followed
16 by a double phenol/chlorophorm extraction (Chomczynski, 1993). The RNA was then treated
17 with DNase I for 20 min at 37 °C in the presence of RNaseOUT™ (Invitrogen). RNA was
18 extracted with phenol/chlorophorm/isoamyl alcohol and additionally purified using RNeasy
19 columns (Qiagen, Hilden, Germany). For each array hybridisation, 1 µg RNA was amplified
20 using the Message Amp II kit (Ambion, Darmstadt, Germany) and 2 µg resulting aRNA was
21 labeled with NHS-activated Cy-3 or Cy-5 dyes (Amersham Bioscience). RNAs from two
22 independent biological replicates were analysed and four technical replicates were performed.
23 The TIGR *A. nidulans* microarrays were provided by the National Institute of Allergy and
24 Infectious Diseases, National Institutes of Health, USA. The polymer-coated glass arrays
25 contained 11,481 70-mer oligonucleotide probes spotted in duplicate, providing 98% genome

1 coverage of 10,701 predicted genes. The slides were prehybridised in 5× SSC, 0.1 % SDS,
2 1 % BSA for 1 h at 65 °C. After washing in distilled water and isopropanol the slides were
3 dried in nitrogen stream. 1.5 µg (ca. 130 pmol) of each labeled RNA was hybridised to the
4 slides using the Agilent ‘60-mer Oligo Microarray Processing’ protocol. The slides were
5 washed and dried in a nitrogen stream. Following hybridisation, spot intensities were
6 determined using an Agilent G2505B microarray scanner.

7

8 ***Data analysis***

9 Microarray intensity data were extracted using the ‘Automatic Image Processing for
10 Microarrays’ software (Katzner *et al.*, 2003). Normalization of the raw intensity data was
11 performed with a non-linear loess regression method. Differentially expressed genes were
12 identified by an ANOVA fixed effects model (Landgrebe *et al.*, 2004). Normalization and
13 statistical computation was performed for two independent data sets derived from a high gain
14 and a low gain scan. ‘Contrasts’ refer to log₂ normalized intensity ratios between wild type
15 and *ΔcsnE* samples. Genes with log₂ ratios ≥ 3.0 and adjusted p values ≤ 0.01 were regarded
16 as significantly regulated and genes with log₂ ratios ≥ 2 and adjusted p values ≤ 0.01 were
17 regarded as moderately regulated. The data are deposited in NCBI's Gene Expression
18 Omnibus;

19 <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=pluhlwwaocgqmpa&acc=GSE22442>

20 and were generated conforming to the MIAME guidelines. Gene probes were annotated and
21 assigned to functional categories manually by homology searches via translated nucleotide
22 BLAST algorithm (blastx). Each gene probe was assigned to one functional category only.

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24 ***Immunoblotting***

25 15-20 µg of crude extracts were separated by SDS-PAGE, electroblotted and hybridized with

1 rabbit anti-calmodulin binding protein epitope tag (Upstate/Milliore, Schwalbach, Germany),
2 mouse anti-GFP (Clontech-Takara Bio Europe, Saint-Germain-en-Laye, France) or rabbit
3 anti-actin C11 peptide (Sigma-Aldrich, Steinheim, Germany) antibodies.

4

5 ***Two-dimensional electrophoresis, protein visualization and image analysis***

6 70 µg of protein were treated with chloroform and methanol, pelleted and resolved in
7 rehydration buffer to a final volume of 350 µl. The mixture was used for the hydration of IPG
8 strips, followed by the multistep focusing protocol using the IPGphor (GE Healthcare Life
9 Sciences, Munich, Germany).

10 After the first dimension, the individual strips were equilibrated for 60 min. The second
11 dimension was performed on homogeneous polyacrylamide gels, which were silver stained
12 and analyzed with PDQuest (Bio-Rad Industries, München, Germany). Extracts of three
13 independent cultures were analyzed with at least two replicate gels for each.

14

15 ***In gel digestion and nano-ESI-MS***

16 Tryptic peptides were separated by water-acetonitrile gradients on Dionex-NAN75-15-03-
17 C18-PM columns on an ultimate-nano-HPLC system (Dionex, Sunnyvale, CA, USA). Online
18 ESI-MS/MS2 spectra were generated on an LCQ-DecaXP^{plus} mass spectrometer (Thermo
19 Finnegan, San Jose CA, USA).

20

21 ***Summary of metabolite analysis***

22 Untargeted metabolic fingerprinting was performed by Ultra Performance Liquid
23 Chromatography (UPLC) coupled with time-of-flight mass spectrometry (TOF-MS).
24 Sterigmatocystin extracts were analysed by thin layer chromatography. Psi factor
25 composition was analysed by gas chromatography mass spectrometry (GC MS). Isolated

1 orsellinic acid derivatives were analysed by HPLC. Details are described in supplementary
2 material.

3

4 **Supplementary information**

5 *A.nidulans* strains (Table S8), plasmids (Table S9) primers (Table S10), complete molecular
6 biology, protein extraction, electron microscopy, chemical extraction and analysis procedures
7 as well as full characteristics of the metabolites produced by the *A. nidulans* Δ *csnE* strain are
8 listed in the supplementary information.

9

10

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1 **Figure Legends**

2

3 **Fig. 1.** Fungal CsnE/CSN5 protein expression during development.

4 A. *A. nidulans* development shown schematically and in light microscopy.

5 B. Protein expression of TAP-CsnE during development. Crude extracts of strain AGB381
6 carrying a functional TAP-CsnE and CpeA-sGFP were analyzed by Western blot. CpeA-
7 sGFP served as development-specific control.

8 C. Protein expression of TAP-CsnE in strains defective in other CSN subunits during
9 development. Protein levels of TAP-CsnE were monitored in strain AGB252 (*TAP-csnE*),
10 AGB253 ($\Delta csnA$) or AGB254 ($\Delta csnD$).

11

12 **Fig. 2.** CSN-dependent transcriptome during fungal development.

13 A. Light microscopy. Hyphal morphologies of wild type and $\Delta csnE$ *A. nidulans* grown
14 vegetatively (V14 and V20) are similar, Sexual development (S48) leads to formation of
15 nests (white arrows) in both strains; only the mutant will later be blocked at the primordia
16 stage. During asexual development (A48), mutant and wild type look similar, but only the
17 mutant will later develop sexual nests, which are inhibited in wild type.

18 B. Transmission electron microscopy images of wild type and *csnE* mutant. *A. nidulans*
19 $\Delta csnE$ mutant and wild type cell wall thickness is similar at the time points of transcriptome
20 analysis.

21 C. Transcriptome analysis summary. Genes with differences of \log_2 ratio ≥ 3.0 were used for
22 categorisation. The 'fungal' category denotes significantly conserved or unique fungal genes
23 with no assigned function; 'other' denotes genes with assigned function of other categories.

24

1 **Fig. 3.** CSN-dependent fungal proteome during acquisition of developmental competence.

2 A. Crude extracts from *A. nidulans* wild type (wt) or $\Delta csnE$ strains grown vegetatively for 20
3 hours (V20) were separated by 2D gel electrophoresis.

4 B. *A. nidulans* proteins from spots in (A) identified by mass spectrometry. Best cross
5 correlation coefficients (X_{corr}), number of different peptides (pep), comparison to known
6 proteins and their proposed function are given.

7

8 **Fig. 4.** Comparison of CSN effect on gene expression by Northern and microarray analysis.

9 A. Northern hybridization. Ribosomal protein S15 (AN5997) was used as internal control.
10 *mutA*, *ppoC* and *catD* transcriptional activation fails in the $\Delta csnE$ strain at S48, V14/V20 and
11 A48, respectively. Genes for an F-box protein (AN2505) and tyrosinase (AN5311) are
12 misactivated in the mutant strain at A48/S48 and V14/V20, respectively. The AN7893
13 oxygenase gene expression is activated and AN5046 defensin gene expression inhibited at all
14 times in $\Delta csnE$ strain. V: vegetative growth, A: asexual development (in light), S: sexual
15 development (in the dark).

16 B. Transcript quantification. Normalized \log_2 ratios of expression levels of genes of (A)
17 derived from 2 biological and 2 technical replicates of microarray experiments. The time
18 point with the highest expression value of each gene was set to 1 (100 %).

19

20 **Fig. 5.** $\Delta csnE$ strain has impaired resistance to oxidative stress and produces an unusual
21 secondary metabolite pattern.

22 A. Sensitivities of wild type (wt) and $\Delta csnE$ strains to the indicated concentrations of
23 menadione, diamide, GSH or GSSG.

1 B. Secondary metabolite production of wild type and $\Delta csnE$ under grown in NH_4 -medium
2 (NH_4) or NO_3 -medium (NO_3) in dark (d) or light (l). 1-5 indicate the identified orcellinic acid
3 derivates.

4 C. Structures of the identified substances: 1: orcinol; 2: violaceol II; 3: violaceol I; 4: cordyol
5 C; 5: diorcinol.

6

7 **Fig. 6.** CSN-dependent metabolite fingerprinting during fungal development.

8 A. 1D-SOM matrix after metabolite-based clustering of the non-polar extracts analyzed with
9 negative ionization mode of 443 marker candidates. Intensity profiles of 20 prototypes
10 (horizontal dimension) of wild type and $\Delta csnE$ were compared; timepoints correspond to
11 these of the transcriptome analysis. Colours of matrix elements represent average intensity
12 values.

13 B. Intensity profiles of six selected ST biosynthesis intermediates of prototype 7 which
14 accumulate only in the $\Delta csnE$ mutant at A48 and to a lower extent at S48.

15 C. Accumulated intermediates of the sterigmatocystin (ST) pathway. The indicated ST
16 intermediates (red boxes) and *stc* transcripts (green boxes) were increased in the $\Delta csnE$
17 mutant at A48.

18

19 **Fig. 7.** Amounts of the PpoA and PpoC-derived psi factors 8-HOE, 8-HOD and 10-HOD
20 produced in the $\Delta csnE$ mutant and the wild type.

21 A. 10-HOD levels, (B) 8-HOD, and (C) 8-HOE levels during different developmental stages
22 of the $\Delta csnE$ mutant and the wild type. Psi factors were purified using reversed phase HPLC
23 and detected by GC MS. Measurement was performed for both strains at all developmental
24 timepoints.

25

1 **Fig. 8.** CSN and fungal development. Vegetative CSN activities (CSN_{VEG}) are required to
2 protect the fungus against oxidative stress. They are prerequisite for proceeding through
3 development beyond the primordial state, where an internal oxidative stress signal is
4 required. Development-specific CSN activities (CSN_{DEV}) inhibit the sexual pathway by light.
5 They shift the ratio of psi factors which are fungal hormones. They also activate cell wall
6 degrading enzymes to restructure the cell wall for development and control the production of
7 protective secondary metabolites.

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1 **Table 1.** Ratios of oleic to linoleic acid-derived psi factors in the *A. nidulans* $\Delta csnE$ mutant
 2 and the wild type at different developmental timepoints

Time point	strain	8-HOE:8-HOD ¹
V14	$\Delta csnE$	1 : 6,2
	wild type	1 : 4,8
V20	$\Delta csnE$	1 : 8,2
	wild type	1 : 19,6
A48	$\Delta csnE$	1 : 18,7
	wild type	1 : 2,7
S48	$\Delta csnE$	1 : 15,1
	wild type	1 : 8,3

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4 ¹8-HOE: 8-hydroxy oleic acid; 8-HOD: 8-hydroxy linoleic acid

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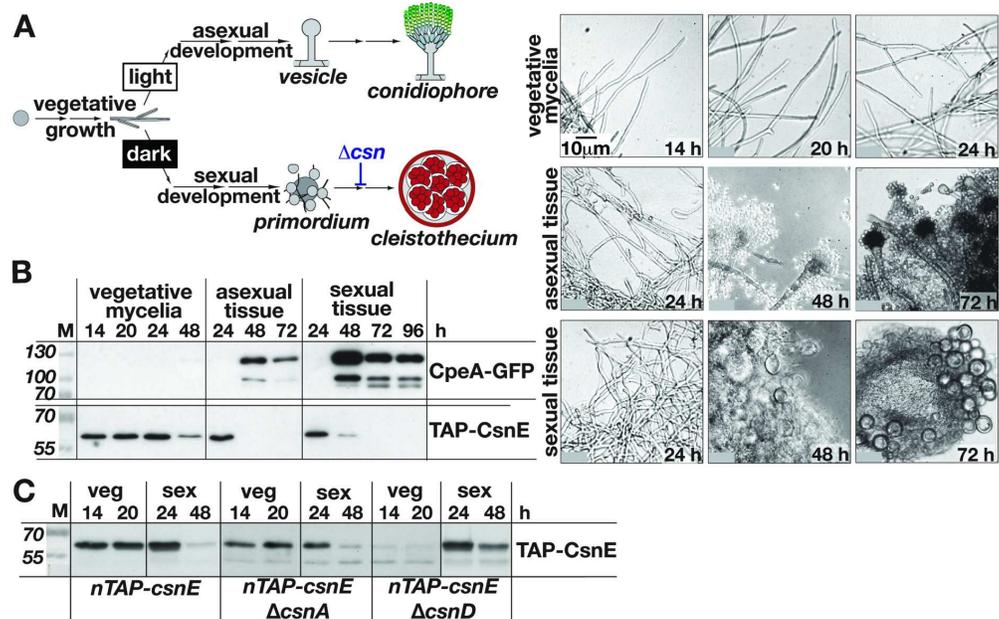


Figure 1
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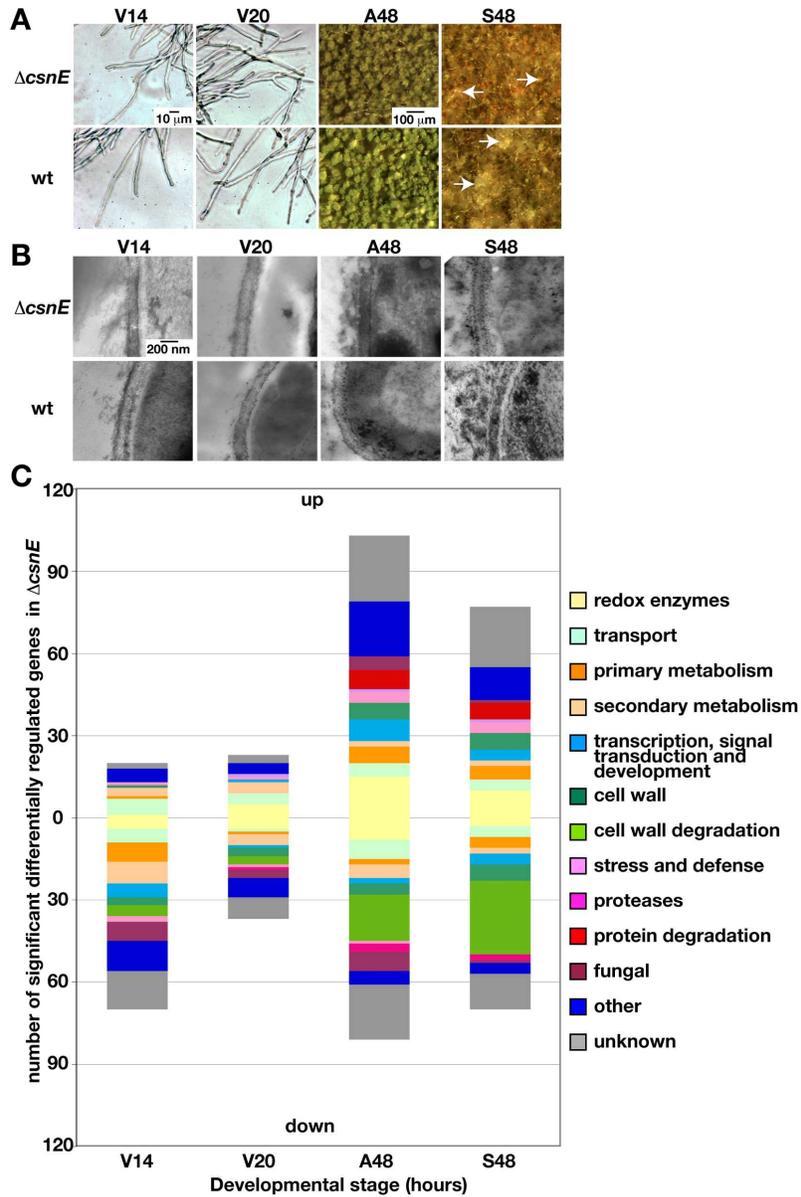


Figure 2
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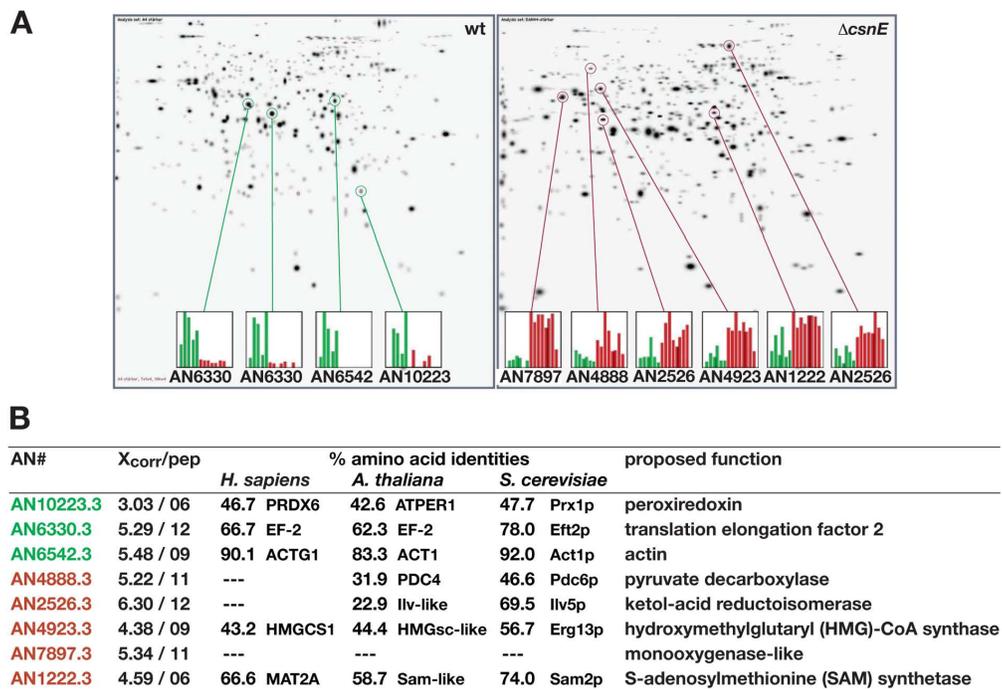


Figure 3
160x111mm (300 x 300 DPI)

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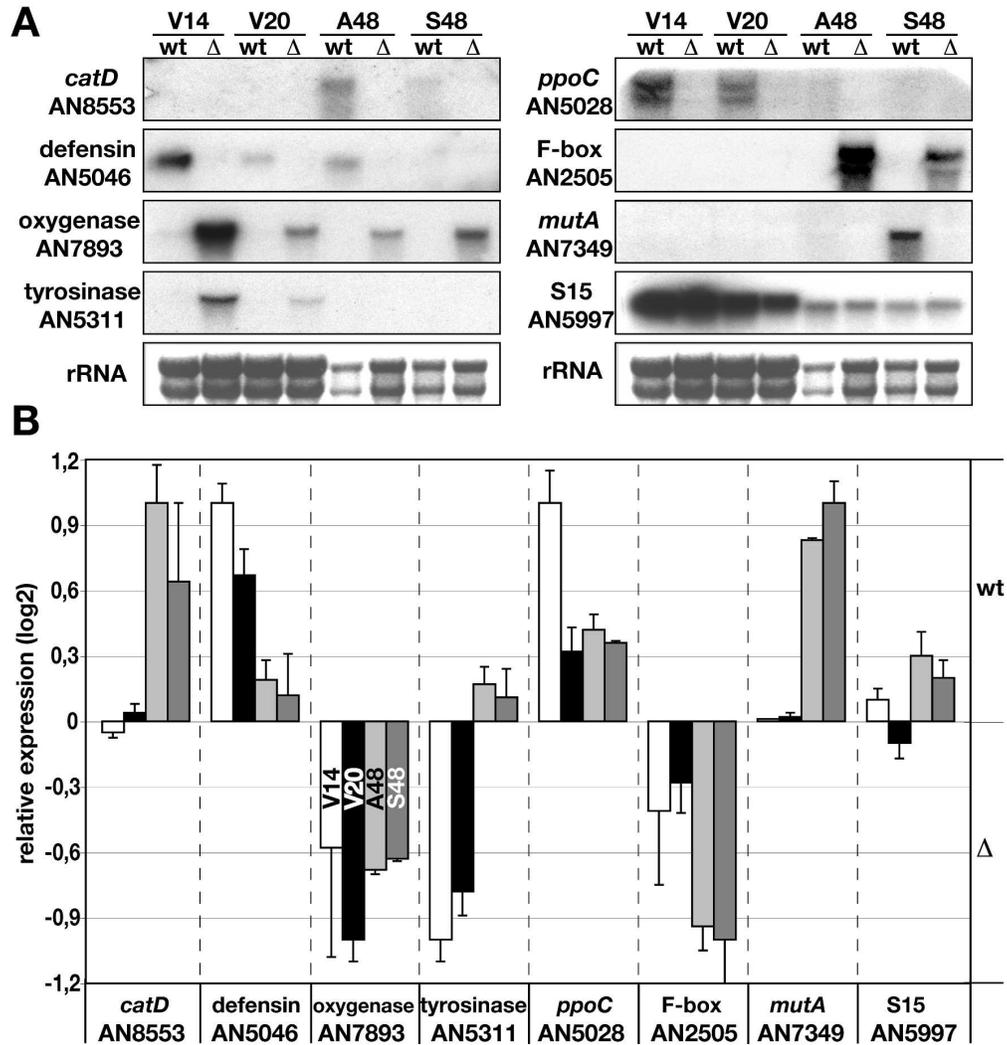


Figure 4
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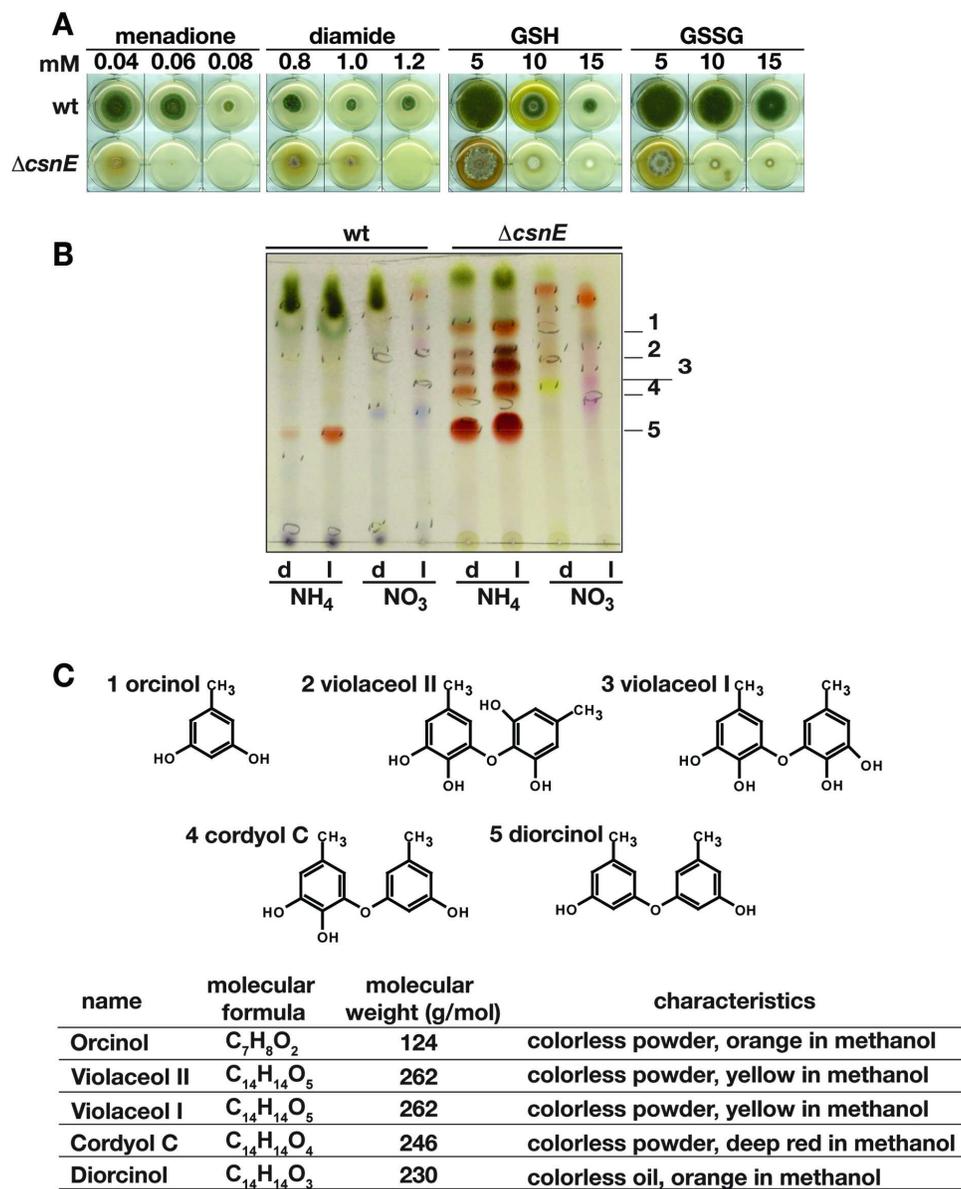


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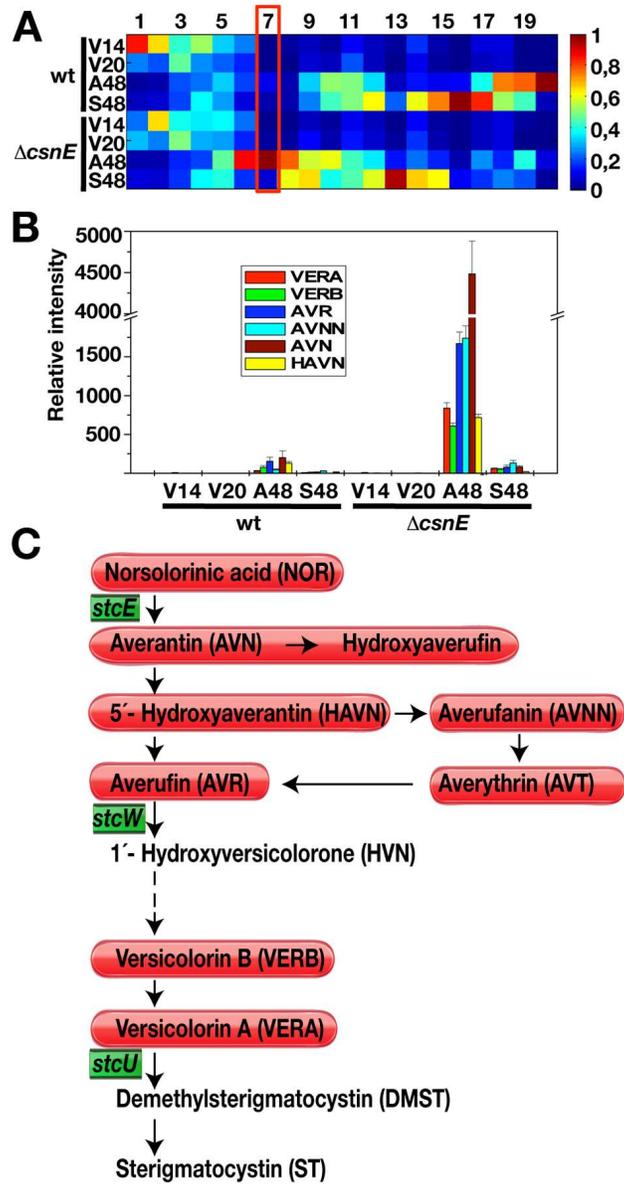


Figure 6
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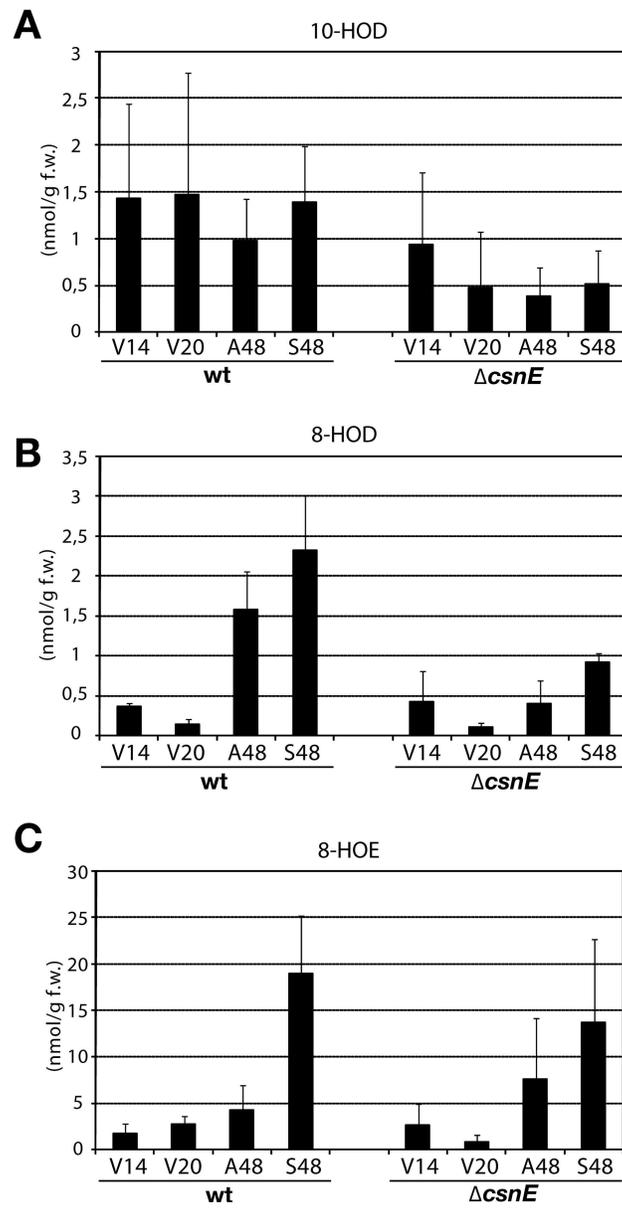


Figure 7
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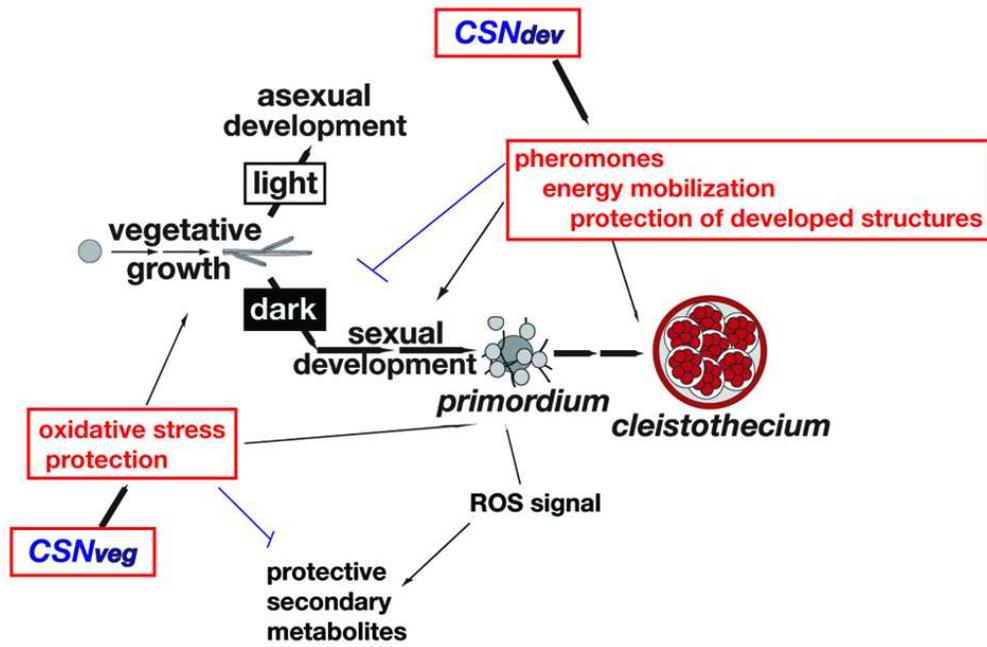


Figure 8
80x52mm (300 x 300 DPI)

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